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SARS-COV-2 ORF9b Dysregulate Fibrinogen and Albumin Genes in a Liver Cell Line

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Abstract

Background: Individuals experiencing severe cases of Coronavirus Disease 2019 (COVID-19) exhibited elevated fibrinogen levels and decreased albumin levels, potentially linked to the presence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) proteins. Consequently, our study endeavors to examine the impact of SARS-CoV-2 ORF9b on the expression of fibrinogen and albumin genes within the Hep-G2 cell line.

Methods: In this study, the Hep-G2 liver cell line was utilized alongside the plasmid pcDNA3.1 hyg+ containing ORF9b from the SARS-CoV-2 strain originating in Wuhan. Transfection procedures were executed, and the transfected cells were selected utilizing hygromycin B. Validation of ORF9b expression was conducted through SYBR green-based real-time PCR, and the expression of the Fibrinogen α (FGA), Fibrinogen β (FGB), Fibrinogen γ (FGG), and Albumin (ALB) genes was quantified using the same method.

Results: The real-time PCR analysis revealed a significant upregulation of fibrinogen genes—α (P=0.03), β (P=0.02), and γ (P=0.029) in Hep-G2 cells containing ORF9b compared to control cells. Furthermore, the findings indicated a markedly lower expression level of albumin in Hep-G2 cells harboring ORF9b compared to the control cells (P=0.028).

Conclusion: The findings suggest that SARS-CoV-2 ORF9b could potentially influence the course of SARS-CoV-2 infection by triggering the expression of α, β, and γ fibrinogen gene chains while suppressing the albumin gene. Further investigations are warranted to validate these observations across various SARS-CoV-2 strains exhibiting differing levels of pathogenicity.

Keywords: Fibrinogen, ORF-9b protein, SARS-CoV-2, Serum Albumin.

Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), commonly known as the 2019 novel coronavirus (2019-nCoV), was first identified in late December 2019 (1). Coronavirus disease 2019 (COVID-19), a respiratory tract infection caused by SARS-CoV-2, manifests across a clinical spectrum, ranging from asymptomatic cases to acute respiratory distress syndrome (2). The outcome of infection can be impacted by various host factors, such as health status, age, genetic makeup, and virus- related elements, including the genetic composition of the virus (3, 4). SARS-CoV-2 shares a range of proteins with other coronaviruses, including 16 nonstructural proteins (nsp1-16) and four key structural proteins (S, E, M, and N proteins). In addition to these shared proteins, SARS-CoV-2 possesses its own distinct set, comprising ORF3a, ORF 3b, ORF6, ORF7a, ORF7b, ORF8a, and ORF9b (5, 6). These specific proteins, designated as accessory proteins due to their perceived non-essential role in virus replication, have been

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highlighted in research for their importance in facilitating virus-host interaction and pathogenesis (6, 7).

The ORF9b protein is encoded by an alternate open reading frame situated within the N gene (8). Previous studies have revealed the presence of antibodies targeting ORF9b in the serum of individuals who recovered from either SARS-CoV or SARS-CoV-2 (9, 10). Through its interaction with the adaptable adapter translocase of outer mitochondrial membrane protein 70 (TOM70) within the mitochondria, this protein has been shown to significantly inhibit the production of IFN-I (11).

A study suggests that the proteins NSP4 and ORF9b encoded by SARS-CoV-2 synergistically induce the release of mitochondrial DNA (mtDNA) (12). Previous research has underscored the crucial role of mitochondria in regulating essential pathways associated with the pro-inflammatory response and antiviral signaling (13). Circulating mtDNA levels have been proposed as a potential biomarker for evaluating both pro-inflammatory reactions and the severity of COVID-19, as indicated by a separate study (14). However, a comprehensive understanding of the molecular mechanisms underlying mtDNA release during pathological conditions, particularly in the context of SARS-CoV-2 infection, remains elusive.

Fibrinogen, a crucial protein implicated in blood clotting and thrombosis, serves as a positive acute phase reactant, as it tends to increase during inflammatory responses (15). Observations suggest that individuals with COVID-19 often display heightened fibrinogen levels (16). Autopsies conducted on COVID-19 patients have revealed the presence of fibrin-rich thrombi within pulmonary capillaries and small arteries (17).

Furthermore, research consistently demonstrates a correlation between COVID-19 infection and hypoalbuminemia(18). Albumin, a protein exhibiting negative acute phase reactivity, is known to be downregulated during inflammatory responses, and it plays a critical role in the body's antioxidant defense system. Hypoalbuminemia has been associated with an inflammatory response and an unfavorable prognosis in viral diseases, including COVID-19 (18). The objective of the present investigation was to evaluate the influence of SARS-CoV-2 ORF9b transfection on the expression of the fibrinogen and albumin genes in the Hep-G2 cell line.

Materials and Methods

Cell culture and transfection

The Hep-G2 cell line was employed in this study and cultured using DMEM supplemented with 10% Fetal Bovine Serum (FBS), following standard procedures. For transfection, the plasmid pcDNA3.1 hyg+ containing ORF9b from the SARS-CoV-2 Wuhan strain was used, alongside a control lacking the gene. Transfection protocols were conducted using DNAfectamin (Biobasic, Canada) according to the manufacturer's instructions. To isolate transfected cells expressing ORF9b, 24 hours post-transfection, hygromycin B (Biobasic, Canada) at a concentration of 250 μg/mL was introduced into the cell culture medium for 7 days. After this period, only cells containing the plasmid were survived.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from cell cultures using an RNA isolation kit (Dena Zist, Mashhad, Iran) as per the manufacturer's instructions. The concentration and quality of the extracted total RNA were assessed using spectrophotometry (NanodropTM Spectrophotometer, Thermo Scientific, USA) and gel electrophoresis, respectively. To eliminate plasmid contamination, the total RNA underwent RNAse-free DNAse treatment using Sinaclon from Tehran, Iran. Subsequently, cDNA synthesis was carried out through reverse transcription (RT) following the manufacturer's guidelines, utilizing an EasycDNA Synthesis kit (Parstos, Mashhad, Iran).

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Design of primers and real-time PCR

The process of designing primers was performed by using primer design software, which relied on the NCBI gene database as a reference (refer to Table 1). The SYBR Green chemistry was utilized in real-time PCR to validate the expression of the ORF9b and to measure the expression levels of the albumin (ALB Gene ID: 213), fibrinogen α (FGA Gene ID: 2243), fibrinogen β (FGB Gene ID: 2244), and fibrinogen γ (FGG Gene ID: 2266) genes. The QuantStudio 3™ real-time PCR system, manufactured by Applied Biosystems located in Grand Island, New York, United States, was utilized to assess gene expression. The Beta-actin gene was utilized as the reference gene (19). Each reaction employed a final volume of 15 µL, comprising 2× Master Mix Green (Ampliqon Inc., Denmark) at 7.5 µL, 1 µL of cDNA, 0.4 µL of each primer (10-pmoL concentration), and 5.7 µL of water. Cycling parameters consisted of an initial denaturation phase at 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 15 seconds, and annealing/extension at 60 °C (or 58 °C for the fibrinogen γ gene) for 1 minute. Confirmation of amplification for a single target in each gene test reaction was achieved, with the absence of primer dimer formation confirmed by analyzing melting curves of all amplifications. Product dimensions were determined using gel electrophoresis to run real-time PCR products, yielding products of 192, 163, 229, and 174 bp for the genes encoding albumin, fibrinogen α, fibrinogen β, and fibrinogen γ, respectively.

Table 1. Sequences of primers were used in real-time PCR.

Gene Name	Primer	Sequences (5'-3')
Albumin	Forward	TCAGTATCTTCAGCAGTGTCCAT
	Reverse	GCACAGCAGTCAGCCATT-3´
Fibrinogen A chain	Forward	CAGCCAATAACCGTGATAATACCT
	Reverse	ATGTCCACCTCCAGTCGTT
Fibrinogen β chain	Forward	ACTTAGCACTCTCCACTTAGCA
	Reverse	CCGACAGCATTAACTCAAGCATA
Fibrinogen γ chain	Forward	ACAGTGCCAGGAACCTTG
	Reverse	CATCCATTTCCAGACCCATCG
Beta actin	Forward (19)	GCCTTTGCCGATCCGC
	Revers (19)	GCCGTAGCCGTTGTCG

Verification of ORF9b expression

After extracting RNA from the transfected cells, it underwent treatment with RNase-free DNase (Sinaclon, Tehran, Iran) to remove any plasmid contamination. Real-time PCR was employed to confirm the expression of ORF9b. The experimental negative control entailed using RNA subjected to DNase treatment.

Data analysis

The $2^{-\Delta\Delta Ct}$ values derived from real-time PCR experiments underwent statistical scrutiny utilizing the Mann-Whitney U test in GraphPad Prism version 5.0. A significance level of 0.05 was applied to ascertain statistical significance.

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Results

Gene expression analysis of fibrinogen chains and albumin in Hep-G2 harboring ORF9b in comparison with control

Real-time PCR analysis confirmed the expression of the ORF9b in Hep- G2 cells containing ORF9b. Moreover, the transcriptional activity of the fibrinogen α chain was notably elevated in Hep-G2 cells harboring ORF9b compared to the control group (P=0.03) (Fig. 1).

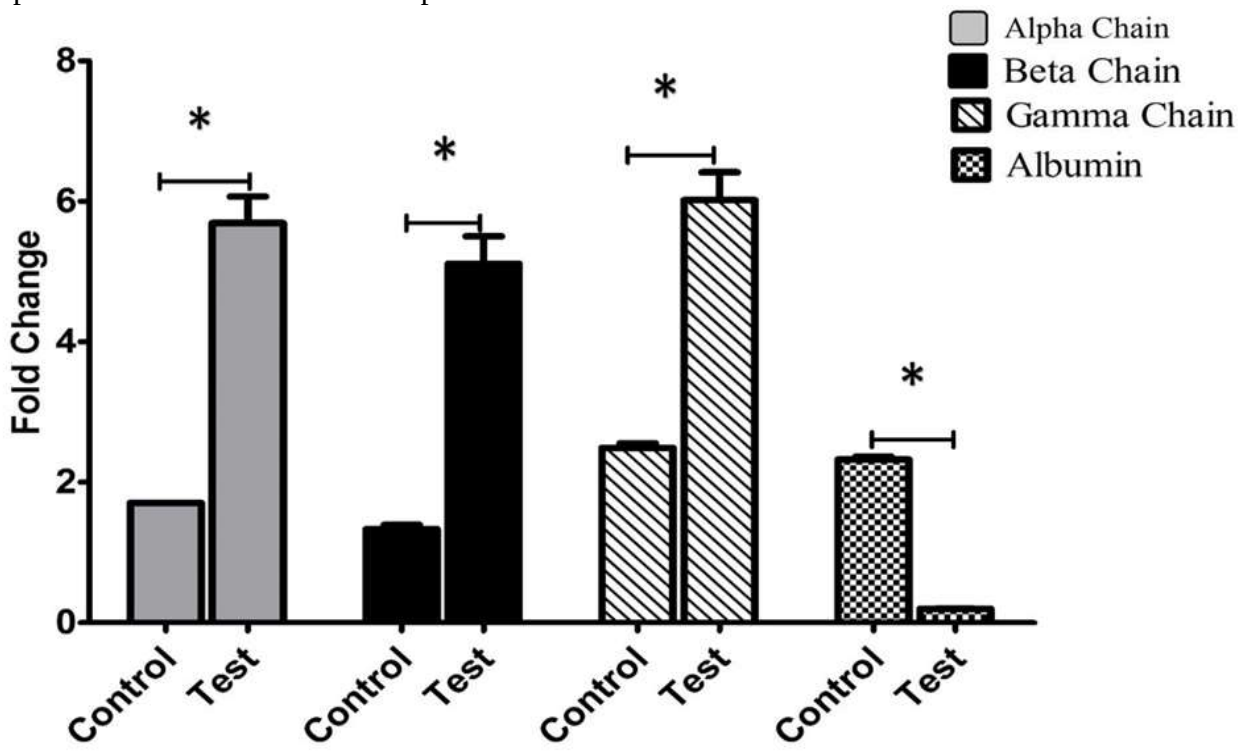

Fig. 1. Gene expression in Hep-G2 harboring ORF9b in comparison with control. Transcriptional activity of the fibrinogen α chain was significantly increased in Hep-G2 cells expressing ORF9b compared to the control group **(**P = 0.03).

Discussion

The present study showed an upregulation in the expression of three fibrinogen genes after the introduction of the ORF9b from SARS-CoV-2 into the Hep-G2 cell line. Fibrinogen, a liver-produced glycoprotein known for its anti-infective properties, can, however, pose risks when overexpressed during acute inflammatory responses, potentially leading to coagulation and thrombosis (20). This phenomenon is particularly relevant in COVID-19 cases, where elevated fibrinogen levels correlate with increased inflammation, disease severity, and ICU admissions (21). Research by Long et al. indicates that abnormal fibrinogen levels are associated with heightened mortality risk in COVID-19 patients, serving as a prognostic indicator for critical illness onset (22). Additionally, Sui et al. observed elevated fibrinogen levels in severe COVID-19 cases (21). Furthermore, the study by Rezaei-Tavirani et al. highlights the significance of fibrinogen chains, specifically FGA, FGB, and FGG, in predicting COVID-19 prognosis and fatality (23). The liver's hyperactive response during the acute inflammatory phase of COVID-19 leads to the secretion of various inflammatory proteins, including fibrinogen (24, 25). The fibrinogen function in patients with acute COVID-19 and clot formation were studied (21, 26). Investigating the coagulation processes and molecular pathways involved in fibrinogen induction offers potential avenues for managing and treating COVID-19 patients (23).

In COVID-19 patients, SARS-CoV-2 exerts a direct impact on liver function, often

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resulting in hepatic degeneration, as evidenced by common abnormalities in liver enzymes (27). Several studies have established a strong correlation between circulating mitochondrial DNA (mtDNA) levels and the severity of COVID-19 (28, 29). Additionally, research indicates that circulating mtDNA can serve as a predictive marker for disease severity and the ensuing pro-inflammatory response (12). NSP4 and ORF9b, both encoded by SARS-CoV-2, have been identified as proteins that facilitate the release of mtDNA (12). Faizan et al. provided the initial molecular evidence demonstrating that the release of mtDNA from the inner membrane is a coordinated process regulated by Induced myeloid leukemia cell differentiation protein (MCL1) (12). MCL1 overexpression at a genetic level serves to safeguard the inner membrane from damage and the subsequent release of mtDNA (12). Verification of MCL1's role in orchestrating the formation of inner membrane vesicles was observed in ORF9b-expressing cells (12). ORF9b's influence in promoting vesicle formation within the inner membrane was evident in transfected airway epithelial cells, where direct interaction with MCL1 was noted, thereby restraining MCL1 accumulation (12). The extracellular release of mtDNA has the potential to impact neighboring cells by activating the TLR9 signaling pathway, subsequently initiating downstream signaling cascades that trigger the production of chemokines and pro-inflammatory cytokines (PICs) (29-31). Stress and circulating cell-free mitochondrial DNA (Cf-mtDNA) derived from the plasma of COVID-19 patients was found to robustly induce a pro-inflammatory response in primary airway epithelial cells, ultimately leading to cellular demise (12). Previous investigations have demonstrated a significant positive correlation between the release of cf-mtDNA and pro-inflammatory mediators in clinical blood samples from COVID-19 patients, mirroring findings observed in our study (28, 29).

In our study, another gene demonstrating a significant alteration was albumin, with its transcript exhibiting downregulation in the Hep-G2 cell line following ORF9b expression. Previous investigations have substantiated that individuals afflicted with COVID-19 undergo an exacerbated oxidative stress response, resulting in elevated levels of reactive oxygen species (ROS), with albumin playing a pivotal role in fortifying the body's antioxidant defense mechanism (32-34). Throughout the SARS-CoV-2 pandemic, consistent reports have linked the clinical presentation, symptoms, and outcomes of COVID-19 with hypoalbuminemia (35, 36). Notably, recent research has highlighted hypoalbuminemia as an independent risk factor for mortality, with hypoalbuminemic patients exhibiting a 6.394 times higher risk of mortality compared to those with normal albumin levels (35). Moreover, concurrent hypoalbuminemia correlates with prolonged hospitalization and increased mortality rates (37). Furthermore, hypoalbuminemia serves as a robust early predictor of in-hospital mortality in COVID-19 cases, irrespective of age, inflammatory markers, or comorbidities (18). The utility of low albumin levels as a reliable prognostic biomarker for identifying severe illness early on, thereby aiding clinicians in optimal patient management decisions, has been emphasized (36, 38, 39). Additionally, independent of the Charlson-Age Comorbidity Index, hypoalbuminemia exhibits a strong association with adverse outcomes in COVID-19 patients. Aziz et al. conducted a meta-analysis underscoring the link between severe COVID-19 cases and hypoalbuminemia (38). Moreover, higher albumin levels upon admission were linked to significantly fewer adverse outcomes, including venous thromboembolism (VTE), acute respiratory distress syndrome (ARDS), ICU admission, and readmissions within 90 days post-screening (39). Anber et al. reported markedly lower average serum albumin concentrations in both severe and non-severe COVID-19 patients compared to healthy controls. They proposed that serum alanine transaminase (ALT) activity serves as the most effective biomarker for

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distinguishing between non-severe and severe patients, while albumin concentration remains an exceptional discriminator between patients and controls (40).

In summary, the findings suggest that the ORF9b strain of SARS-CoV-2 from Wuhan could potentially impact the course of SARS-CoV-2 infection by upregulating the α, β, and γ chains of fibrinogen genes. Additionally, SARS-CoV-2 ORF9b may play a role in the pathogenesis of SARS-CoV-2 infection by inhibiting the expression of the albumin gene. Further investigations are warranted to validate these findings across different strains of SARS-CoV-2 and to explore the potential of ORF9b protein as a target for medication development.

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Ethics committee code
Ethical approval is not applicable, because this article does not contain any studies with human or animal subjects.

Conflicts of interest
The authors state no conflict of interest.

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